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Oncogenic Activation of the Lck Protein Accompanies Translocation of the *LCK* Gene in the Human HSB2 T-Cell Leukemia

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The tyrosine protein kinase p56^{lck} transduces signals important for antigen-induced T-cell activation. In transgenic mice, p56^{lck} is oncogenic when overexpressed or expressed as a mutant, catalytically activated enzyme. In humans, the *LCK* gene is located at the breakpoint of the t(1;7)(p34;q34) chromosomal translocation. This translocation positions the β T-cell receptor constant region enhancer upstream of the *LCK* gene without interrupting the *LCK* coding sequences, and a translocation of this sort occurs in both the HSB2 and the SUP-T-12 T-cell lines. We have found that, although the level of the p56^{lck} protein in HSB2 cells is elevated approximately 2-fold in comparison with that in normal T-cell lines, total cellular tyrosine protein phosphorylation is elevated approximately 10-fold. Increased levels of phosphotyrosine in HSB2 cells resulted from mutations in the *LCK* gene that activated its function as a phosphotransferase and converted it into a dominant transforming oncogene. The oncogenic p56^{lck} in HSB2 cells contained one amino acid substitution within the CD4/CD8-binding domain, two substitutions in the kinase domain, and an insertion of Gln-Lys-Pro (QKP) between the SH2 and kinase domains. In NIH 3T3 fibroblasts, three of these mutations cooperated to produce the fully oncogenic form of this p56^{lck} variant. These results suggest that mutation of *LCK* may contribute to some human T-cell leukemias.

The lymphocyte-specific tyrosine protein kinase, p56^{lck}, is expressed predominantly in T cells (13, 30, 53), where it is physically associated with the T-cell transmembrane proteins CD4 and CD8 (43, 45, 52). During T-cell activation, CD4 and CD8 bind to the major histocompatibility complex glycoprotein (class II or class I, respectively) on an antigen-presenting cell, bringing p56^{lck} in close physical proximity to the T-cell receptor (16). Binding of the antigen-major histocompatibility complex to CD4 and the T-cell receptor subunits triggers the rapid phosphorylation of a set of T-cell proteins on tyrosine (20). p56^{lck} is thought to mediate some of these phosphorylations, because the addition of antibodies that cross-link cell surface CD4 induces tyrosine phosphorylation of p56^{lck} and activates its phosphotransferase activity in vitro (19, 27). Genetic evidence likewise indicates that enhancement of T-cell activation by CD4 is mediated through p56^{lck}. Deletion of the cytoplasmic domain of CD4 that binds p56^{lck} abrogates the ability of CD4 to enhance antigen-induced interleukin 2 (IL-2) production in CD4-dependent cells (18, 33, 46), and T cells expressing activated p56^{lck} are hypersensitive to stimulation through the T-cell receptor (1) while those lacking functional p56^{lck} are defective in inducing the transient increase in intracellular free calcium that accompanies antigenic stimulation through the T-cell receptor (48). Therefore, p56^{lck} performs a pivotal role in T-cell activation.

The phosphotransferase activity of p56^{lck} is regulated in a manner similar to that of the other members of the Src family of tyrosine protein kinases (Src, Yes, Lck, Lyn, Fgr, Fyn, Blk, and Hck; reviewed in reference 15). The carboxyl-terminal catalytic domain of p56^{lck} becomes autophosphorylated at Tyr-394 (29) upon transient activation of p56^{lck} by addition of antibodies against CD4 or CD8 (27) or upon permanent activation of p56^{lck} by oncogenic mutations that replace Tyr-505 with other residues (5, 29). By contrast, phosphorylation of Tyr-505 negatively regulates catalytic function in vivo (5, 29), possibly through the binding of its phosphorylated form to the SH2 domain located in the amino-terminal half of p56^{lck} (25, 31). Replacement of Tyr-505 with phenylalanine activates phosphotransferase activity in vivo, induces transformation of NIH 3T3 fibroblasts (5, 29), and potentiates activation of T cells (1, 28).

In spite of the simple mechanism by which mutation of p56^{lck} at tyrosine 505 creates an oncogenic kinase, neither p56^{lck} nor any other Src family member has been observed as an oncogene in human cancer. Here we report that p56^{lck} expressed by the human T-cell leukemia line, HSB2, has become oncogenic as a consequence of novel mutations within the *LCK* coding sequence. HSB2 cells have an early T-cell phenotype (CD4$^-$ CD8$^-$), express the cytotoxic T-cell antigen Ly9, and were derived by direct implantation of buffy coat cells from a patient with acute lymphoblastic leukemia into a newborn Syrian hamster, which subsequently acquired lymphomas (2, 3). The mechanism of p56^{lck} catalytic activation is unique in HSB2 because it does not involve mutation of the carboxyl-terminal regulatory tyrosine residue. HSB2 p56^{lck}, nevertheless, exhibits the hallmarks of an activated tyrosine kinase: constitutive autophosphorylation in vivo, excessive substrate phosphorylation, and induction of morphological transformation in fibroblasts.

Interestingly, HSB2 cells also contain a t(1;7)(p34;q34) translocation that fuses the proximal promoter of one *LCK* allele on chromosome 1 with the constant region enhancer of the β T-cell receptor from chromosome 7 (10, 51). Because transient activation of p56^{lck} accompanies normal T-cell activation and because transient repression of *LCK* transcription normally follows T-cell activation (36, 37), we suggest that constitutive activation of the p56^{lck} tyrosine kinase may persistently repress *LCK* transcription, and we propose that translo-

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cation with the β T-cell receptor enhancer could abrogate this negative regulation and therein allow continuous expression of an oncogenic p56^{lck} variant in HSB2 cells.

MATERIALS AND METHODS

Immunoblot analysis, immunoprecipitations, and peptide mapping. Total cellular extracts of human cell lines were prepared and analyzed for their content of p56^{lck} and phosphotyrosine-containing proteins by immunoblotting as described elsewhere (23), by using either affinity-purified rabbit antibodies against phosphotyrosine (23) or a rabbit anti-p56^{lck} serum prepared by immunization with a *trpE-lck*-encoded fusion protein expressed in *Escherichia coli* (22). Immunoprecipitation of p56^{lck} was performed from cells labeled biosynthetically for 16 h with 100 μCi ^{35}S-methionine and -cysteine (NEN) or 1 mCi $^{32}P_i$ as described elsewhere (22). p56^{lck} was isolated from polyacrylamide gels (7). Peptides generated by cleavage of ^{35}S-labeled p56^{lck} with chymotrypsin or ^{32}P-labeled p56^{lck} with trypsin were resolved by electrophoresis at pH 8.9 in the first dimension and by chromatography in the second dimension (21).

Phosphoamino acid analysis. Immunoprecipitated p56^{lck} was purified by electrophoresis through polyacrylamide gels containing 10% sodium dodecyl sulfate, transferred to Immobilon-P (Millipore), and subjected to autoradiography (24). The strip of Immobilon containing p56^{lck} was excised and subjected to hydrolysis in 5.7 N HCl for 60 min at 110°C (24). The hydrolysate was lyophilized and analyzed by two-dimensional electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension (21).

Cloning of HSB2 Lck. mRNA was isolated from the cytosol of HSB2 cells and purified twice by adsorption to oligo(dT) Sepharose (Pharmacia). Synthesis of the first strand was accomplished by using Moloney murine leukemia virus reverse transcriptase (Promega) after denaturation with methylmercuric hydroxide. Second-strand synthesis was accomplished by using DNA polymerase I, RNase H, and *E. coli* DNA ligase. The 3′ overhangs were blunted by using T4 DNA polymerase, and *Eco*RI-*Not*I adapters were ligated to the DNA (Invitrogen). After addition of linkers, the dephosphorylated cDNA was fractionated by electrophoresis through a 1.0% agarose gel, and the population of cDNAs larger than 1.5 kbp was selected for ligation into *Eco*RI-cut lambda gt10 (Stratagene). The ligated cDNA was packaged by using Gigapack (Stratagene) and plated on an Hfl strain of C600. Recombinant clones containing *LCK* sequences were identified by probing with a fragment from the human *LCK* cDNA clone, YT16 (26). *LCK* cDNAs were cloned into pBSK− (Stratagene), and both strands were sequenced by using a panel of 12 oligonucleotide primers and Sequenase (United States Biochemicals).

Expression of *LCK*. The entire *LCK* coding sequence encompassed by the *Eco*RI-to-*Stu*I fragments of the normal Lck clone YT16 (26); the normal Lck clone HK28 (38); the HSB2 Lck clone A1; and each of the variants of HK28 containing one, two, or three of the four mutations found in the A1 clone from HSB2 cells were cloned into the *Eco*RI site of the retroviral vector pLXSN. Helper-free retroviruses encoding each Lck variant were generated by cotransfection of COS cells with the pLXSN-Lck expression vectors and the packaging-defective ecotropic proviral clone pMLV, designated SV-ψ$^-$-E-MLV, as described elsewhere (34). Helper-free virus encoding normal murine p56^{lck} or F505 Lck (catalytically activated) was used as a control (41). After 48 h, supernatants from transfected COS cells were used to infect NIH 3T3 cells. Infected NIH 3T3 cells expressing p56^{lck} were selected by growth in medium containing 600 μg of G418 (effective drug) per ml.

Nucleotide sequence accession number. The GenBank accession number of the sequence determined in this study is U07236.

RESULTS

HSB2 cells contain highly elevated levels of phosphotyrosine-containing proteins. The possibility that human leukemias might contain activated tyrosine protein kinases was investigated by subjecting total cellular extracts of leukemia cell lines to immunoblotting with antiphosphotyrosine antibodies (Fig. 1A, Anti P-tyr). One cell line, HSB2 (Fig. 1A, anti P-tyr, lane 4), reproducibly demonstrated a significantly higher level of staining, and intense staining of proteins with molecular masses of 52 to 62 kDa, which migrated close to the position of overexpressed p56^{lck} in LSTRA cells (lane 5, arrow) was observed. T-cell lines Molt 4D (lane 1), Jurkat (lane 2), CEM (lane 3), HPB.MLT, RPMI 8402, JM, and Molt 3; B-cell lines IM9, Raji, Ramos, BJAB, Namalva, P3HR1.1, and Daudi; an erythroleukemia cell line HEL; and the myelomonocytic cell line U937 did not exhibit a substantial increase in total cellular phosphotyrosine (subset represented in Fig. 1). Phosphoamino acid analysis of total cellular protein, labeled biosynthetically with $^{32}P_i$, demonstrated that HSB2 cells contained 13.5 times as much phosphotyrosine as did control B-JAB cells (0.63% of total acid-stable phosphoamino acids versus 0.047%).

p56^{lck} is activated, but not substantially overexpressed, in HSB2 cells. Because one of the major phosphotyrosine-containing proteins in HSB2 cells comigrated with p56^{lck}, we examined whether p56^{lck} might be overexpressed or activated in HSB2 cells. Immunoblotting (Fig. 1A, Anti Lck) revealed that there was less than a twofold increase in the abundance of p56^{lck} in HSB2 cells (lane 4) compared with levels in control Molt 4D (lane 1), Jurkat (lane 2), and CEM (lane 3) T cells. Biosynthetic labeling (Fig. 1B) confirmed that there was less than a twofold increase in the abundance of p56^{lck} in HSB2 cells (lane 8 and 9) compared with levels in control CEM (lanes 6), Jurkat (lane 7), and JM (lane 10) T cells. However, extended electrophoresis of these immunoprecipitates revealed that ^{35}S-methionine-labeled p56^{lck} in HSB2 cells (lanes 8 and 9) exhibited a slower electrophoretic mobility than p56^{lck} isolated from control cells (lanes 6, 7, and 10). To determine whether p56^{lck} was one of the proteins in total HSB2 cell lysates that stained heavily with antiphosphotyrosine antibodies, it was isolated by immunoprecipitation from unlabeled cells and subjected to immunoblotting with antiphosphotyrosine antisera (Fig. 1B, Anti P-tyr). Purified p56^{lck} from HSB2 cells (lanes 8 and 9) stained much more intensely than did p56^{lck} from control CEM (lane 6), Jurkat (lane 7) and JM (lane 10) T cells. The altered mobility of p56^{lck} in HSB2 cells, as well as its strong staining by antiphosphotyrosine antibodies, suggested that it differed qualitatively from normal p56^{lck}.

Phosphotryptic peptide analysis was performed to determine the manner in which tyrosine phosphorylation of p56^{lck} differed in HSB2. HSB2 p56^{lck} was found to be extensively phosphorylated at both its autophosphorylation site, Tyr-394, and its carboxyl-terminal regulatory site, Tyr-505 (Fig. 2A). p56^{lck} from Jurkat was not phosphorylated substantially at Tyr-394 but was phosphorylated at Tyr-505 and at other sites known to contain phosphoserine (Fig. 2A). Analysis of phosphoamino acids in each p56^{lck} protein demonstrated that HSB2 p56^{lck} also contained an elevated ratio of phosphotyrosine to phosphoserine compared with that isolated from

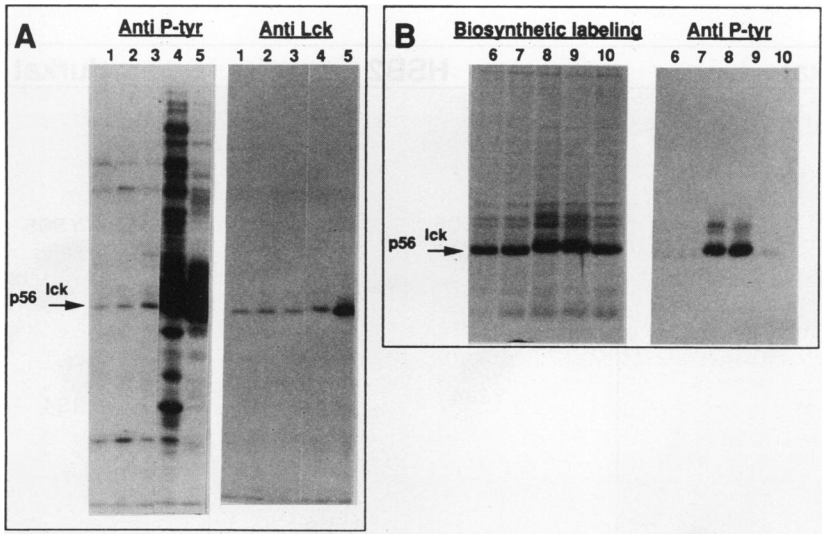

FIG. 1. Elevated protein tyrosine phosphorylation and an altered form of p56^{lck} are detected in HSB2 T cells. (A) Immunoblots of total T-cell proteins stained with antibodies against phosphotyrosine (P-tyr) or Lck. Lanes 1, Molt-4D human T cells; lanes 2, Jurkat human T cells; lanes 3, JM human T cells; lanes 4, HSB2 cells; lanes 5, LSTRA mouse B cells. (B) Immunoprecipitations of Lck from T-cell lines labeled biosynthetically or from a parallel dish of unlabeled cells that were subsequently analyzed by immunoblotting with antiphosphotyrosine antibodies. Samples in lanes 6 to 10 were subjected to electrophoresis twice as long as those in lanes 1 to 5 of panel A. Lanes 6, CEM human T cells; lanes 7, Jurkat human T cells; lanes 8 and 9, HSB2 cells; lanes 10, JM human T cells.

Jurkat cells (Fig. 2A, inset at lower right). The fact that HSB2 p56^{lck} is extensively phosphorylated at its autophosphorylation site suggests that it is persistently activated.

Chymotryptic peptide analysis demonstrates that the majority of HSB2 p56^{lck} is physically different from that encoded by Jurkat T cells. Activation of p56^{lck} in HSB2 cells could result from intrinsic mutations in the *LCK* gene or from activating mutations of a gene encoding an upstream regulator of the p56^{lck} kinase. To determine whether p56^{lck} in HSB2 cells was mutant, two-dimensional chymotryptic peptide maps of p56^{lck} from HSB2 and Jurkat T cells were compared. Maps of ^{35}S-methionine- and ^{35}S-cysteine-labeled p56^{lck} encoded by HSB2 cells contained a new peptide designated "a" and exhibited an 80 to 90% reduction in the intensity of peptides b to e (Fig. 2B). Peptide a was not a normal peptide shifted by phosphorylation, because it did not comigrate with any $^{32}P_i$-labeled chymotryptic peptide (data not shown). These data suggested that the predominant form of p56^{lck} in HSB2 cells is mutant.

Predicted amino acid sequence of HSB2 p56^{lck} contains four mutations. An HSB2 cDNA library was prepared, one full-length and two partial *lck* cDNAs were isolated, and both strands of all three clones were sequenced. In comparison with the normal sequence of human *LCK* clone HK28 (38), HSB2 p56^{lck} contained three point mutations and one insertion of 3 amino acids (Fig. 3). Resequencing of HK28 across each predicted HSB2 mutation site confirmed that each change in HSB2 *LCK* represented a true difference from the normal HK28 *LCK* sequence. All mutations occurred near a methionine or cysteine residue and should affect the mobility of a chymotryptic peptide during chromatography and electrophoresis. Mutation 1 changed codon 28 from GTC (valine) to CTC (leucine). Val-28 lies 5 residues to the carboxy terminus of two cysteines that are absolutely required for the binding of p56^{lck} to CD4 or CD8 (44). Mutation 2 inserted nine nucleotides, CAGAAGCCC (encoding glutamine, lysine, and proline), at amino acid 230, exactly between the SH2 and kinase domains (54). In normal p56^{lck}, a tandem sequence of glutamine, lysine, and proline (QKPQKP), encoded by CAGAAG CCC-CAGAAGCCG, represents amino acids 231 to 236. At the DNA level, the 9-bp sequences encoding each QKP repeat differ only in the ninth position. The sequence of the additional QKP insert is identical to the first 9-bp sequence, creating the sequence CAGAAGCCC-CAGAAGCCC-CAGAAGC CG. This insert adds 0.353 kDa to the mass of p56^{lck} and may be the cause of the slight reduction in the electrophoretic mobility of HSB2 p56^{lck} (Fig. 1B). Within the QKPQKP sequence of normal p56^{lck}, the internal KPQ sequence is conserved in six of eight Src kinase family members, and the internal proline residue is absolutely conserved (Fig. 3B). Mutations 3 and 4 occurred within the kinase domain, changing codon 353 from GCA (alanine) to GTA (valine) and codon 447 from CCG (proline) to CTG (leucine). Ala-253 is absolutely conserved within the Src family, whereas Pro-447 is conserved in only three of the eight members (Fig. 3B). At positions that are mutant in HSB2 Lck, the sequences of all normal *LCK* clones, including three different human isolates (YT16 [26], λgHlck1 [42], and HK28 [38]) and one normal mouse isolate (30), encode the same conserved amino acids. This indicates that the mutations in HSB2 Lck do not represent common polymorphisms.

The abundance of the mutant Lck transcript in HSB2 cells was examined by using PCR to amplify Lck-specific cDNA sequences from total HSB2 cDNA. Sequence analysis of PCR products encompassing mutations 2 and 4 revealed that more than 80% of cDNAs contained each mutation. Therefore, the mutant transcript represents the major form of *lck* mRNA in HSB2 cells.

Mutant HSB2 p56^{lck} is an activated, oncogenic kinase in vivo. To examine the oncogenicity of HSB2 Lck, helper-free virus expressing normal human Lck (YT16), HSB2 Lck, normal mouse Lck, and an oncogenic variant of mouse Lck encoding phenylalanine instead of tyrosine at amino acid 505 (F505 Lck) were used to infect mouse NIH 3T3 cells. Infected cells were isolated by growth in G418. Expression of both HSB2 and F505 Lck induced morphological transformation in

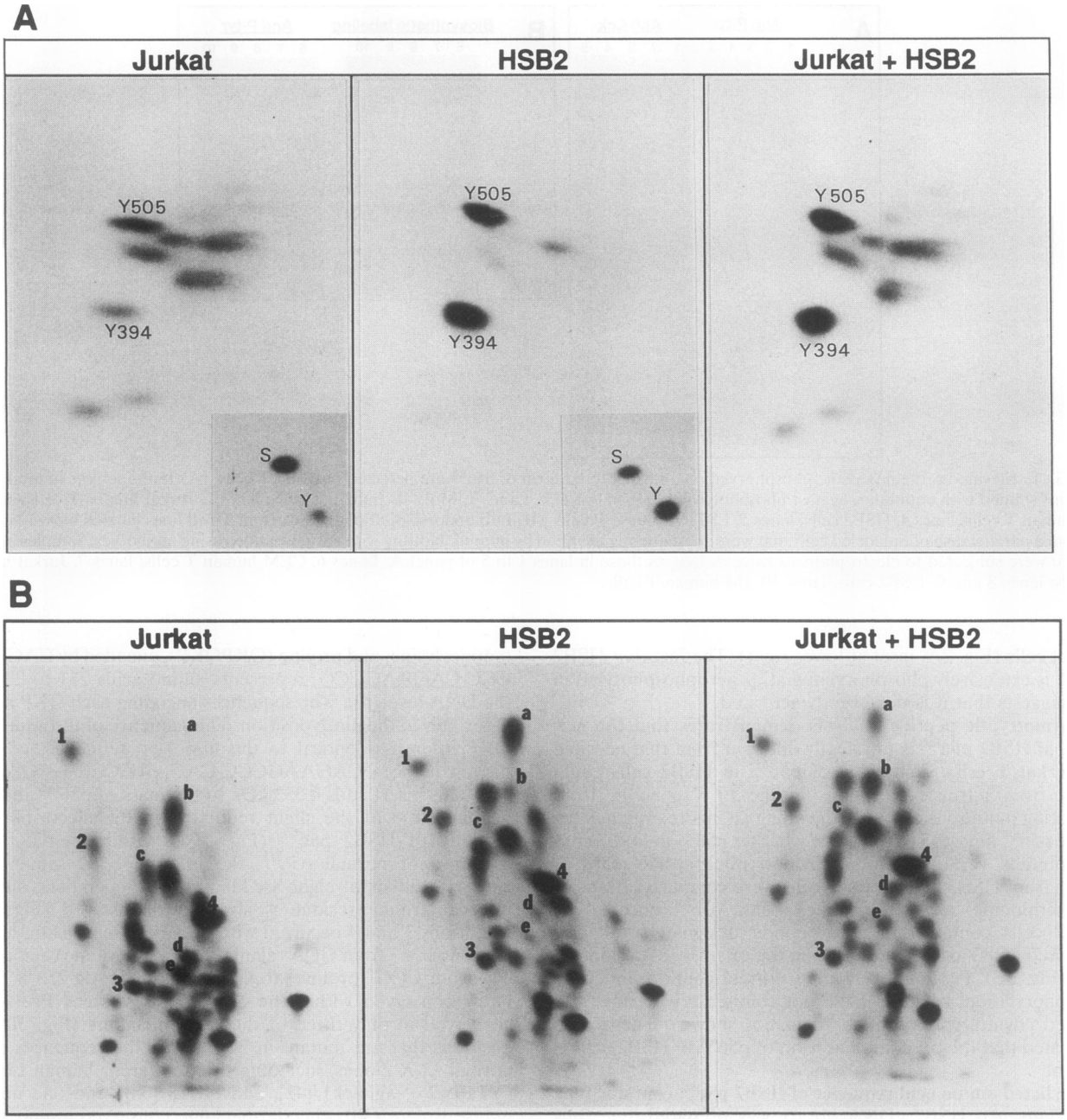

FIG. 2. Peptide maps of p56^{lck} isolated from Jurkat or HSB2 T cells. (A) Phosphotryptic peptide maps of p56^{lck} from Jurkat and HSB2 T cells, labeled in vivo with $^{32}P_i$. Jurkat + HSB2, a mixture of equivalent radioactive amounts of labeled tryptic peptides from Jurkat p56^{lck} and HSB2 p56^{lck}. Insets represent two-dimensional phosphoamino acid analysis of p56^{lck} labeled in vivo with $^{32}P_i$ and isolated by immunoprecipitation. S, phosphoserine; Y, phosphotyrosine. (B) Chymotryptic peptide maps of p56^{lck} labeled in vivo with ^{35}S-methionine and ^{35}S-cysteine. Peptides 1 to 4 are those referred to in the text. Peptide a is uniquely found in HSB2 p56^{lck}, and peptides b to e exhibit a substantially reduced abundance in HSB2 p56^{lck}.

G418-selected colonies and induced growth in soft agar (Fig. 4; Table 1). Expression of neither the normal mouse Lck nor the normal human Lck induced these transformation properties (Table 1), despite a recent report claiming that the YT16 clone of Lck is oncogenic (32). The transforming ability of HSB2 Lck was not due to its overexpression in these infected fibroblasts, because anti-Lck immunoblots of total cell extracts (Fig. 5B) demonstrated that cells transformed by HSB2 Lck (lane 3) contained approximately the same amount of Lck as did nontransformed cells expressing either normal human Lck (lane 2) or normal mouse Lck (lane 4). Instead, transformation by HSB2 Lck and F505 Lck was associated with elevated levels of tyrosine protein phosphorylation. Antiphosphotyrosine immunoblotting (Fig. 5A) revealed that the level of tyrosine protein phosphorylation produced by expression of HSB2 Lck (lane 3) was much greater than that produced by comparable levels of normal human Lck (lane 2) or normal mouse Lck (lane 4) and was at least as great as that produced by the same

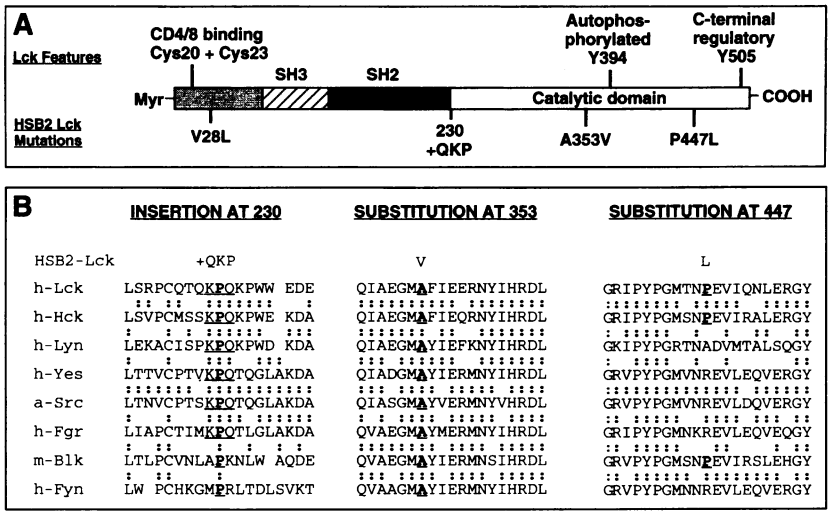

FIG. 3. Mutations in HSB2 p56[lck] and conservation of surrounding sequences among the Src family of tyrosine kinases. (A) Features of normal p56[lck] and sites of mutations in HSB2 p56[lck]. (B) Conservation of sequences of the Src family tyrosine kinases surrounding mutated residues 230, 353, and 447 of HSB2 p56[lck]. References are as follows: human Lck (h-Lck), 38; h-Hck, 40; h-Lyn, 54; h-Yes, 49; avian Src (a-Src), 50; h-Fgr, 35; murine Blk (m-Blk), 17; h-Fyn, 44.

level of oncogenic F505 Lck (lane 5). This suggested that the specific activity of HSB2 Lck had increased to a level at least as great as that of F505 Lck. A clone of NIH 3T3 cells derived from an agar colony expressing HSB2 Lck contained even more total cellular phosphotyrosine than did NIH 3T3 cells transformed by the oncogenic p160[gag-abl] tyrosine protein kinase (Fig. 5A, lane 6 versus 7). We conclude that HSB2 Lck is a catalytically activated, oncogenic tyrosine kinase.

Mutations 2 to 4 of HSB2 Lck cooperate to produce the oncogenic properties of HSB2 Lck in NIH 3T3 fibroblasts. To define the contribution that each of the four mutations made to the transforming ability of HSB2 Lck, we constructed variant Lck proteins containing subsets of the four mutations and evaluated their ability to induce focus formation on NIH 3T3 fibroblasts. The titers of helper-free viral stocks were determined on the basis of their ability to transmit G418-resistant growth to cells in liquid culture, and the transformation efficiency induced by each form of Lck is expressed as the number of foci formed in unselected cultures that were infected with 10,000 G418 resistance units of virus (Fig. 6A). To prove that all Lck variants were equally expressed, parallel plates of cells infected by each virus were grown in G418, and

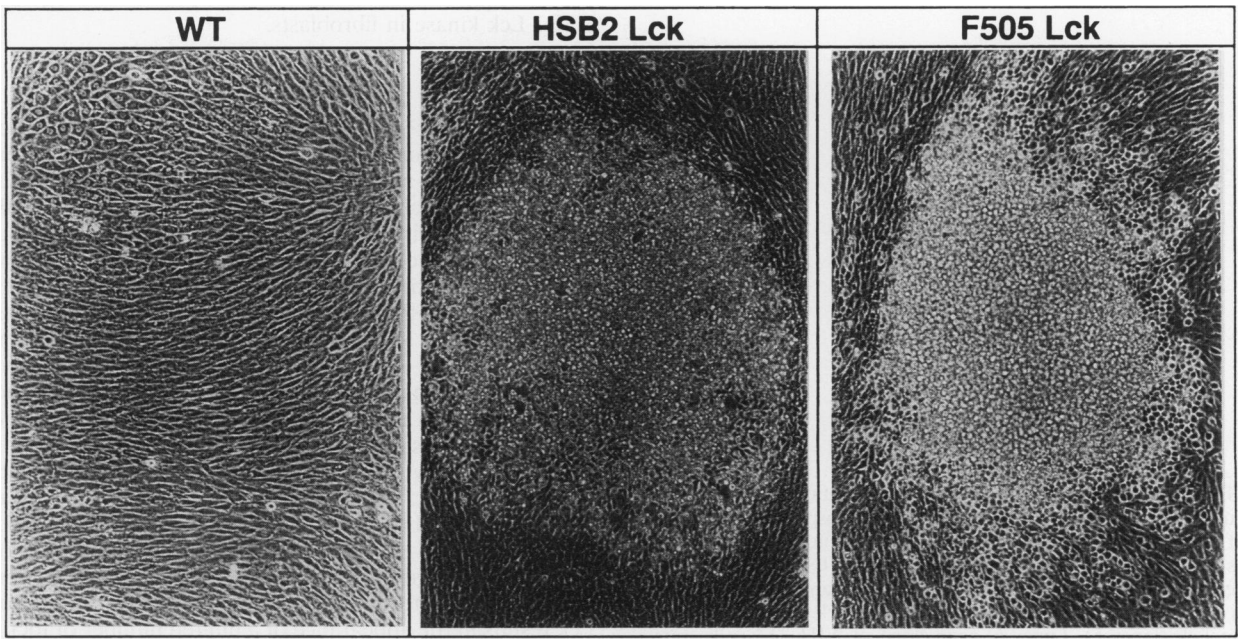

FIG. 4. Focus formation induced by HSB2 p56[lck]. Morphology of NIH 3T3 fibroblasts infected by virus encoding normal mouse Lck (WT), mutant HSB2 Lck (HSB2 Lck), or mutant mouse Lck that is biochemically activated by substitution of tyrosine 505 with phenylalanine (F505 Lck).

TABLE 1. Transforming abilities of HSB2 Lck in NIH 3T3 fibroblasts

Lck[a]	No. of transformed colonies[b]/ no. of G418-resistant colonies (%)	No. of agar clumps[c]/10^4 infected cells (%)
Normal mouse	0/152 (0.0)	0 (0.0)
F505 mouse	16/44 (36.4)	320 (3.2)
Normal human[d]	0/120 (0.0)	0 (0.0)
HSB2 human	76/146 (52.1)	2,800 (28.0)

[a] Expressed by a retrovirus vector.
[b] Cells transformed by both F505 and HSB2 Lck exhibited the same fusiform, refractile morphology.
[c] Colonies scored in this assay consisted of 10 or more clumped cells. However, many clumps contained more than 50 cells, and the distribution of the sizes of agar colonies for cells expressing F505 Lck was essentially the same as that for cells expressing HSB2 Lck.
[d] YT16.

the Lck content of these cells was examined by immunoblotting with an anti-Lck serum (Fig. 6B). The abundance of the different Lck proteins in cells infected by each virus was very similar, indicating that the number of transformed foci produced by infected cells that were grown in the absence of G418 should reflect the oncogenic potential of each mutation or combination of mutations. In this assay, expression of HSB2 Lck (designated HSB2-M1234) produced more than 250 times as many foci as did normal human Lck (clone HK28), which yielded a background of 9 small foci. Lck proteins containing mutations 1, 3, or 4 (HSB2-M1, HSB2-M3, and HSB2-M4, respectively) exhibited no transforming potential. By contrast, the introduction of mutation 2 alone in Lck (HSB2-M2) created a moderately transforming version of Lck that produced the same-size foci as did HSB2-M1234 but was 10-fold less efficient than HSB2-M1234. Mutations 3 and 4 cooperated to produce a weakly transforming Lck protein (HSB2-M34) that induced the growth of small foci. However, combining mutation 2 with mutations 3 and 4 produced an oncoprotein (HSB2-M234) that was as transforming as wild-type HSB2-M1234. Because the addition of mutation 1 did not increase the transforming potential of either HSB2-M2 or HSB2-M234, mutation 1 does not contribute in an obvious manner to the transforming ability of HSB2-M1234 in NIH 3T3 fibroblasts. We conclude that mutations 2 to 4 cooperate to deregulate the HSB2 Lck kinase in fibroblasts.

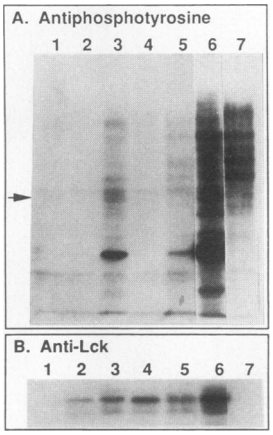

FIG. 5. Levels of tyrosine protein phosphorylation in cells infected by viruses expressing $p56^{lck}$ variants. (A) Antiphosphotyrosine immunoblot of total cell lysates; (B) anti-Lck immunoblot of total cell lysates. Total G418-selected populations of NIH 3T3 cells were infected by an empty vector (lanes 1), normal human Lck (YT16 clone) (lanes 2), HSB2 Lck (lanes 3), normal murine Lck (lanes 4), or F505 murine Lck (lanes 5). Lanes 6, agar clone of NIH 3T3 cells transformed by HSB2 Lck; lanes 7, NIH 3T3 cells transformed by the activated oncogenic tyrosine protein kinase $p160^{gag\text{-}abl}$. Arrow, $p56^{lck}$.

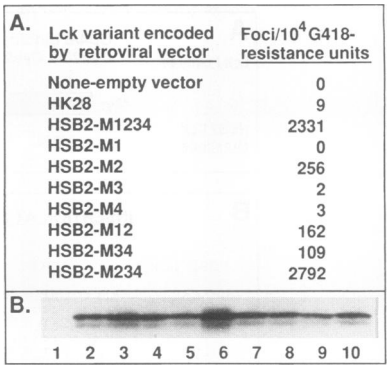

FIG. 6. Dissection of the oncogenic effects of each of the four mutations in HSB2 Lck. (A) Helper-free retroviruses encoding Lck variants containing combinations of the four HSB2 Lck mutations were tested for their ability to induce focus formation on NIH 3T3 cells. The titer of each retrovirus was assessed in parallel plates by growth of infected cells in G418, and the transforming strength of each Lck is expressed in terms of the number of foci per 10,000 G418 resistance units. Control viruses were HK28 (expressing normal human Lck), HSB2-M1234 (expressing HSB2 Lck containing all four mutations), and an empty vector (pLXSN retroviral vector expressing no Lck cDNA). Virus-encoding combinations of the four mutations in HSB2 Lck have the prefix HSB2 followed by an "M" designation which specifies which mutations are contained in the variant Lck. As described in Results, mutation 1 is V-28→L, mutation 2 is an insertion of QKP at position 230, mutation 3 is A-353→V, and mutation 4 is P-447→L. (B) Expression of each variant Lck protein in G418-selected cells detected by immunoblot analysis using anti-Lck antibodies. Cells were infected by virus encoding an empty vector (lane 1), HSB2-M1234 (lane 2), HK28 (lane 3), HSB2-M34 (lane 4), HSB2-M12 (lane 5), HSB2-M234 (lane 6), HSB2-M4 (lane 7), HSB2-M3 (lane 8), HSB2-M2 (lane 9), or HSB2-M1 (lane 10).

DISCUSSION

The *SRC* gene family represents a large pool of potential oncogenes in human malignancies. However, the strongest evidence linking activation of a Src family kinase to human cancer prior to this report is a severalfold activation of the in vitro kinase activity of $p60^{c\text{-}src}$, which occurs as an early event in some human colon carcinomas (8, 11, 12). In this situation, no internal mutations in $p60^{c\text{-}src}$ have been reported, and the in vivo phosphorylation of proteins on tyrosine remains essentially unchanged. Mayer et al. (32) have claimed that the YT16 *LCK* clone (the same clone we used as a control in our experiments), which was isolated from Jurkat cells and sequenced by Koga et al. (26), is oncogenic because transfection of YT16 expression constructs yielded some foci in their experiment. They initially performed this experiment because the sequence of YT16 was incorrectly reported to have a carboxyl-terminal frameshift mutation just prior to Y-505. The correct sequence of YT16 reveals that it encodes the normal Lck carboxyl terminus. Adler and Sefton (4) have demonstrated that transfection of expression vectors encoding normal Lck results in the appearance of a limited number of foci, each of which encodes a variant of $p56^{lck}$ that is activated during transfection by disruption of DNA sequences encoding its

carboxyl-terminal residues. The conclusions of Mayer et al. were drawn without the parallel analysis of known oncogenic and normal forms of p56^{lck}. We suggest that the foci observed by Mayer et al. resulted from mutational activation of p56^{lck} by truncation of normal *LCK* coding sequences during transfection (4).

Here we demonstrate that four mutations are contained in the coding sequence of HSB2 *LCK* and that these mutations catalytically activate p56^{lck} and convert it into an oncoprotein. HSB2 p56^{lck} induced extensive tyrosine protein phosphorylation in NIH 3T3 fibroblasts, which resulted in their transformation, as judged by focus formation and growth in agar. By contrast, expression of normal human p56^{lck} did not result in these cellular or biochemical changes. Two additional normal human Lck clones (YT16 [26] and λgHlck1 [42]), as well as a normal mouse Lck clone (30), encode the same conserved amino acids as does the HK28 Lck clone (38) at the positions that are mutant in the HSB2 Lck clone; therefore, we suggest that these mutations do not represent simple polymorphisms in Lck. We conclude that p56^{lck} encoded by HSB2 T cells is an activated tyrosine protein kinase that is oncogenic in fibroblasts and is likely to stimulate proliferation in HSB2 T leukemic cells.

In NIH 3T3 fibroblasts, mutations 2 to 4 cooperated to produce the fully transformed phenotype, while mutation 1 was dispensable for transformation. In isolation, only mutation 2 produced an oncogenic form of Lck. This form of Lck induced the formation of robust foci; however, the frequency of focus formation was 10-fold lower than that for wild-type HSB2 Lck. Mutation 2 represented the insertion of QKP between the SH2 and kinase domains, and we postulate that this may disrupt the normal intramolecular regulation of catalytic function. SH2 has been postulated to bind the carboxyl-terminal regulatory phosphotyrosine residue and suppress catalytic function (31). The recent crystal structure of SH2 affirms its role in binding phosphotyrosine and delineates its boundaries as residues 127 to 224 of p56^{lck} (54). In p56^{lck}, insertion of QKP occurs at the carboxyl-terminal edge of SH2 at residue 230, extending the sequence QKPQKP to QKPQK PQKP. The KPQ sequence embedded within the normal QKPQKP of p56^{lck} is conserved in six of eight members of the Src family (Fig. 3B) and may form part of a hinge that permits the correct associations between the SH2 and kinase domains. If this were the case, addition of another QKP repeat could skew this alignment, prohibit binding of phosphotyrosine 505 within SH2, and produce a constitutively active enzyme without requiring dephosphorylation of Tyr-505. Although we suggest that mutations 2 to 4 are also required to fully activate p56^{lck} in HSB2 T cells, we are hesitant to draw the conclusion that mutation 1 is unimportant. Mutation 1 occurred at codon 28, which lies 5 residues from two cysteines that are critical for association of p56^{lck} with CD4. This mutation could disrupt the association of p56^{lck} with CD4 or another accessory molecule that normally restricts its activity in T cells and be essential for producing a promiscuous Lck kinase in T cells, while being unnecessary for promiscuity in fibroblasts. It has already been observed that T cells have a dominant mechanism that restricts the activity of F505 Lck while fibroblasts do not (1, 5). In T cells, hyperphosphorylation of cellular substrates by F505 Lck is not constitutive but, rather, occurs only after T-cell activation (1, 27), suggesting that F505 Lck activity is negatively regulated by a normal T-cell mechanism that is released during T-cell activation. The ability of HSB2 p56^{lck} to maintain extensive substrate phosphorylation in HSB2 T cells suggests either that HSB2 p56^{lck} contains a mutation that abrogates its ability to be regulated by this system in HSB2 cells or that HSB2 cells lack this regulatory system. HSB2 cells do not express either CD4 or CD8 (2, 3), and therefore, one possibility is that activated Lck is a promiscuous kinase in HSB2 cells because its activity is not restricted by association with either of these proteins. It will be interesting to directly test whether HSB2 Lck also behaves as an activated kinase in T-cell lines that restrict the catalytic function of F505 Lck. If it does, then mutation 1 could play a significant role in disrupting such regulation.

It is not evident whether the mutations in HSB2 Lck occurred in vivo and played a significant role in the genesis of the HSB2 T-cell leukemia or occurred during the establishment and cultivation of HSB2 cells in tissue culture and were therefore unimportant to the development of this human leukemia. However, if these mutations played a formative role in the HSB2 leukemia, it is interesting that their consecutive occurrence in vivo could have created a series of clonal outgrowths containing a progressively more transforming form of p56^{lck}, which could have been evidenced as a progressively more aggressive leukemia. If mutation of p56^{lck} occurred during the generation of this leukemia in vivo, it could have induced growth either by abrogating interleukin 3 dependence of an early T-cell progenitor, as has been observed for the activated tyrosine kinases encoded by v-*abl* (14, 39) or v-*src* (6), or by an autocrine mechanism by inducing expression of the interleukin 2 gene, which is a direct effect of activated F505 Lck (28). Growth stimulation by HSB2 Lck could enhance the likelihood of secondary oncogenic mutations, such as transcriptional activation of the *TAL-1* helix-loop-helix gene, which occurs in HSB2 cells and in 25% of human T-cell acute lymphocytic leukemias, and may serve to disrupt normal differentiation (9).

Why does the t(1;7)(p34;q34) translocation accompany mutational activation of *LCK* in HSB2 cells? The t(1;7)(p34;q34) translocation in HSB2 cells removes the distal promoter of *LCK* from the proximal promoter and replaces sequences 5′ to the proximal promoter with the T-cell receptor β constant region enhancer (10). If this translocation is essential to the leukemic phenotype, it might indicate that transcriptional deregulation of *LCK* is essential to maintain expression of a mutant p56^{lck} protein. Normally, repression of *LCK* transcription accompanies T-cell activation (36, 37). If activation of normal p56^{lck} transiently down regulates *LCK* transcription, then constitutive activation of p56^{lck} would permanently repress *LCK* transcription. In order to maintain expression of an activated p56^{lck} protein in spite of transcriptional repression of the *LCK* gene, a cooperating mutation that results in constitutive *LCK* expression, such as the translocation with a strong T-cell promoter (T-cell receptor β gene), would be required. Although either order of these two independent mutations could occur in the genesis of an oncogenic *LCK* allele, we would postulate that mutations that abrogate *LCK* gene transcription would precede mutations that activate the Lck kinase. To test whether mutational activation of *LCK* may consistently accompany its transcriptional deregulation, we are now examining whether SUP-T-12 cells, which also contain a t(1;7) translocation, express a catalytically activated variant of p56^{lck}. Our preliminary results suggest that this may be the case, as antiphosphotyrosine antibody staining of total SUP-T-12 cellular extracts indicates that they contain as active a tyrosine kinase as do HSB2 cells (data not shown). Therefore, formation of oncogenic *LCK* genes in human T-cell leukemia may be the first example of an oncogene whose formation requires two independent mutations, one that deregulates gene transcription and a second that activates protein function.

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